SENSITIZATION OF HUMAN OSTEOSARCOMA CELL LINE 143B WITH CALCITRIOL FOR CISPLATIN THERAPY

BY

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Submitted to the graduate degree program in Dietetics and Nutrition and the Graduate Faculty of the University of Kansas in partial fulfillment of the degree requirements for the degree of Master of Science.

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ABSTRACT

Background: Osteosarcoma (OS) is the most predominant bone tumor in individuals between 10-25 yrs of age. Cisplatin is the most widely used chemotherapeutic agent for OS management, it significantly enhances the survival rate. Resistance to the drug, toxicities of high doses and metastasis are the major concerns in the treatment.

Objective: Our primary objective is to investigate effects of calcitriol in combination with cisplatin on the osteosarcoma cell apoptosis, invasion and migration. Our hypothesis is pretreatment of OS cells with calcitriol would sensitize them for cisplatin therapy leading to decrease in concentration for IC_{50}. Secondary objective was to evaluate the effect of calcitriol on migration and invasion of OS cell lines, and the role of matrix metalloproteins (MMPs) in migration/invasion.

Design: Previous findings in our lab suggests, calcitriol act as differentiation agent in human osteosarcoma cell lines 143B and SaOS-2. The dose response of cisplatin with and without calcitriol (100nM) pretreatment was studied in osteosarcoma cell lines 143B-MM, 143-P and control murine osteoblast MC3T3-E1. Cell proliferation and sensitization effects were evaluated by MTS assay. Boyden chamber assay was used for migration/invasion studies. MMP activity was measured using Sensolyte kit and real time PCR for the detecting the expression of MMP-1.

Results: Calcitriol does not sensitize the 143B-P and 143B-MM OS cells for cisplatin therapy. Calcitriol sensitization inhibits the migration of 143B-P cells in matrigel whereas no significant difference was observed in control and calcitriol treated 143B-MM cells. Calcitriol pretreatment does modulate MMP-1expression in 143B-P OS cells.

Conclusion: Calcitriol pretreatment does not sensitize OS cells to chemotherapy but, has an effect on the migration and invasion of 143B-P OS cell line.

ACKNOWLEDGEMENTS

It is a pleasure to thank those who made this thesis possible. First and foremost, I thank Dr. Rama Garimella as this project would not have been possible without her guidance and help in research design and techniques. Special thanks to Linda Griffith, Ph.D and Susan Carlson, Ph.D for being on my thesis committee and for their review and suggestions. I thank Dandan Li for her help in the lab. I appreciate the help extended by Dr. Dileepan, Dr. Anant and Dr. Rowe in allowing me to use the equipment in their lab. Finally, my heartfelt thanks to my family members and friends especially my husband, Uday Kiran without his encouragement and support I would not have been able to be here.

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ABBREVIATIONS

OS: Osteosarcoma

143B-P: 143B parental human OS cell line

143B-MM: 143B metastatic cell lines derived from mice

VDR: vitamin D receptor

1,25D: 1α, 25-dihydroxyvitamin D_3

ATCC: American Type Culture Collection

DMEM: Dulbecco's Modified Eagle's Medium

FBS: Fetal bovine serum

HBSS: Hank's Balanced Saline Solution

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

1α- OHase/CYP27B1: 1α-hydroxylase

24α-OHase: 24α-hydroxylase

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

MMP: Matrix Metalloprotein

INTRODUCTION

Osteosarcoma (OS) is the primary bone malignancy affecting children and adolescents especially from ages 10-20yrs, typically occurs in long bones such as femur, tibia and humerus (1). It develops from blocked differentiation in malignant mesenchymal cells. Microscopically, OS is identified by the presence of rapidly proliferating cells and synthesis of abundant osteoid or un-mineralized matrix (2). There are approximately 400 new cases each year in United States and the frequency of occurrence is higher in boys than girls (3). Incidence of OS in adults is associated with Paget's disease and other risk factors include hereditary disorders, Li-Fraumeni syndrome, retinoblastoma, fibrous dysplasia, ionizing radiations and history of bone disorders (7). Clinical symptoms of OS include pain, swelling, palpable mass, weight loss and pathologic fractures in 10-15% of population. Diagnosed with lab results of elevated alkaline phosphatase or lactate dehydrogenase or both, diagnostic scans such as computerized tomography (CT), radio nucleotide, and positron emitted tomography (PET) are helpful in identifying treatment response and follow up recurrences (9). Current therapy against OS includes surgery and multi-agent chemotherapy regimen with drugs such as doxorubicin, cisplatin, methotrexate and ifosamide; this enhances the overall survival rate by more than 50% (8), but there still remain 33% of OS patients who do not respond to the existing chemotherapy. Despite the advances in treatment since the last two decades, about 30-40% of the cases results in

relapse (5). About 90% relapses culminate in pulmonary/lung metastasis, a common cause of death in OS patients (6). Novel treatment regimens are necessary for the patients who are not responsive to the existing therapies and for those who have recurring OS. Reducing the complications and toxic side effects associated with chemotherapy is a challenging goal in OS treatment or management. Newer treatment strategies include designing new chemotherapeutic agents with less toxicity and high efficacy or use of agents to overcome chemoresistance by sensitizing tumor cells to chemotherapeutic drugs at lower concentrations (7, 8, 9). The key for devising effective treatment modalities for OS include identification of relevant biological targets such as genes and signaling pathways in response to chemotherapeutic drugs (10). Application of drugs such as interferons (INF-α), interleukins, bisphosphonates, monoclonal antibodies to sensitize the OS cells to undergo chemotherapy induced apoptosis is known (11).

Vitamin D has a major physiological role in bone metabolism (12). There is an increasing evidence of the anti-cancer properties of active form of vitamin D, calcitriol [1α,25-dihydroxyvitamin D_3]. Relationship between the status of 25-hydroxyvitamin D3 ($25(OH)D_3$), the circulating form of vitamin D and cancer occurrence is reported in several studies (13). Calcitriol can exhibit anti-neoplastic activity by blocking the cell cycle growth, inducing differentiation or apoptotic genes (14). Calcitriol has potentiating action on anti-cancer effects of

chemotherapeutic agents (15). Calcitriol induces cell differentiation in cancer cells, arrests proliferation and suppresses cell cycle and reduces cancer cell survival time. Differentiation pathways of calcitriol vary in different cancer types. For example, in breast and prostate cancers, calcitriol exerts antiproliferative and apoptotic effects on the cell lines (16).

The anti-neoplastic effects of calcitriol in OS are not well studied. Calcitriol can be a modulator of disease and might act at one or all stages of cancer. Vitamin D receptor (VDR) plays a role in differentiation and anti-proliferative effects of calcitriol in OS by modulating the genes involved in cell cycle, angiogenesis and apoptosis, ligand binding to VDR is essential to mediate the antineoplastic effects of calcitriol (14,16).

Osteosarcoma is not radiosensitive and high doses of chemotherapeutic agents are used for treatment (11). Therapeutic approaches promoting the differentiation may be very effective as OS primarily occurs because of a block in the differentiation of mesenchymal stem cells. Evidence suggests the potentiating activity of calcitriol to the chemotherapeutic agents in various cancers such as prostate, colon, breast and blood cancers (15). Sensitization effect of calcitriol in OS to chemotherapeutic agents needs to be clearly investigated.

JUSTIFICATION:

Previous reports including the studies from our lab suggests calcitriol at 100nM dose can induce differentiation and apoptosis in different OS cell lines (40).

Expression of genes encoding for VDR, 1 α-hydroxylase (1 α OHase) and 24-hydroxylase (24-OHase) in response to calcitriol treatment in the OS cell lines and over expression of 24-OHase in the tissue microarrays were observed in previous experiments in Dr. Garimella's lab. Calcitriol enhanced differentiation in 143B, a highly metastatic; K-ras mutated human OS cell line.

Resistance to chemotherapy is the major concern in OS. Cisplatin resistance might be acquired or intrinsic. Other possible mechanisms for chemoresistance can be genetic mutations, expression of apoptotic genes and proteins and rapid detoxification of the drug *in vivo*. Sensitization of recalcitrant cancer cells to chemotherapy is a potential strategy to overcome the chemoresistance. Sensitization effect of calcitriol on chemotherapeutic response is evident in certain cancer cell lines (57-59). However, it is not known whether such an effect exists in OS. With the known role of calcitriol in bone metabolism and in OS cell lines, sensitization should be beneficial in minimizing the resistance to chemotherapy and resulting in decreased cancer cell viability, invasion and migration. The results obtained from this study, if significant will provide valuable information regarding the role of calcitriol as a chemosensitization agent and elucidate the underlying potential mechanism decreasing the chemotherapeutic doses required. Understanding the mechanism underlying metastasis is necessary to improve the treatment protocol and this reduced the relapse rates, as 90% relapses are due to metastasis.

STUDY OBJECTIVES: The study objectives were:

- Evaluation of the sensitization effect of calcitriol for cisplatin therapy on OS cell apoptosis migration, and invasion.
- Establishing the mechanism for the sensitization comparing the changes between calcitriol treated and untreated cell lines.

RESEARCH QUESTION:

Primary: a) Does calcitriol pre-treatment sensitize OS cells for cisplatin therapy? b) Does calcitriol pre-treatment inhibit migration of OS cells?

Secondary:

a) At what concentration is sensitization achieved?

b) Does the mechanism of sensitization involve modulation of caspases?

c) Is migration regulated via matrixmetalloproteins (MMPs)?

HYPOTHESIS:

Our hypothesis is calcitriol pretreatment will sensitize OS cells to cisplatin, thus decreasing the inhibitory concentration (IC_{50}) for cisplatin. We anticipate, calcitriol will sensitize OS cells to cisplatin via modulation of caspase expression and inhibits migration of cancer cells by down regulation of matrix metalloproteins (MMPs).

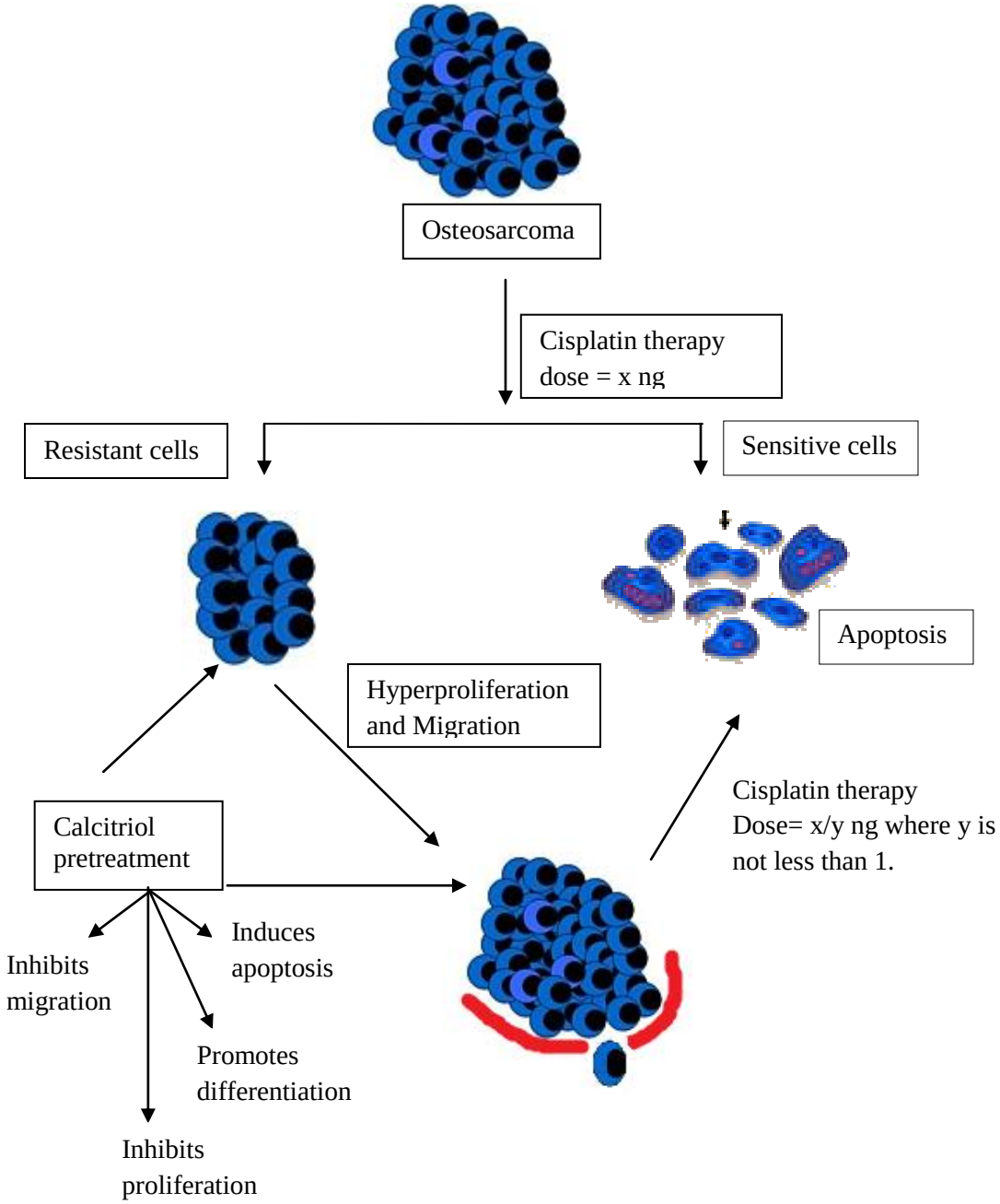


Figure 1: Schematic representation of the hypothesized mechanism of sensitization effect of calcitriol to cisplatin therapy

LITERATURE REVIEW

Osteosarcoma: Osteosarcoma (OS) is the most common primary bone tumor in children and adolescents from ages 10-25yrs with about 500 cases reported annually (1-3, 65). The etiology of OS is multi-factorial, characterized mainly by invasion and metastasis along with other factors (2, 3, 7, 8). The factors associated with OS are environmental such as exposure to radiation and beryllium oxide (2); epidemiological including associations with diseases such as retinoblastoma, Bloom syndrome and genetic impairments such as defects in cell cycle regulation with Rb/p53 mutations (2, 3). Pathogenesis of OS is attributed to aberrations in cell cycle and transcriptional regulations, genomic integrity maintenance, and increased migration, invasion and chemoresistance (3, 4). With the current treatment options, surgery and multi drug chemotherapy survival rate is at 70% (5, 65). Most effective chemotherapeutic agents against OS include high doses of methotrexate plus cisplatin, doxorubicin or ifosfamide. High doses of multiple chemotherapeutic agents are prescribed to increase survival rate. The adverse effects associated with the high doses of chemotherapeutic agents include cardiac toxicity, acute and chronic nephrotoxicity, neurotoxicity, ototoxicity, infertility and malignant secondary tumors (9-12). Progression of OS and metastasis involve neovascularization, proliferation, invasion, apoptosis, resistance and advances to sites such as lungs, kidneys and liver (3, 6, 25, 26). Metastasis is the major causative factor for relapse and other complications

including drug resistance (24). Novel treatment regimens such as therapies including tumor modulating or differentiating agents are very much necessary for patients who develop resistance or for those who are not responsive for the present therapies.

Vitamin D: Vitamin D is a fat soluble vitamin, found in cod liver oil, fish, fortified milk and cereals. It is a secosteroid and functions as a prohormone. Pre-vitamin D_3 or vitamin D_3 obtained from exposure to sun or diet is converted in to 25-(OH)-D_3 in the liver by the enzyme 25-hydroxylase. In the kidney, 25-(OH)-D_3 is hydroxylated to 1,25-$(OH)_2D_3$ by the enzyme 1-α-hydroxylase. The major circulating form is 25-(OH)-D_3 and the functional form is 1,25-$(OH)_2D_3$ (calcitriol). Vitamin D is essential for calcium homeostasis and its deficiency is characterized by inadequate mineralization of bone, leading to acute consequences on bone health such as rickets or osteomalacia (64). Role of vitamin D is well established in the bone metabolism and in various cancers such as breast (15, 22, 59) and prostate (36, 44, 53).

The major physiological function of vitamin D is to regulate serum calcium concentration. This is achieved by increasing intestinal calcium absorption. Vitamin D stimulates osteoclastogenesis from monocyte precursors in the presence of receptor activator of nuclear factor kappa-B ligand **(**RANKL). Activated or mature osteoclasts actively resorb bone mineral and release calcium, thus maintaining blood calcium levels. Parathyroid hormone (PTH) secreted by

chief cells of parathyroid gland, regulates blood calcium and phosphorous levels. When blood calcium levels are low, PTH promotes bone resorption, decreases renal calcium excretion and increases 1-α-hydroxylase levels, increasing calcitriol production. PTH secretions are decreased when blood calcium levels are high. Increased secretions of PTH are often associated with vitamin D deficiency and renal defects (64). Hence, vitamin D deficiency causes secondary hyperparathyroidism results in loss of phosphorus by PTH (16). Low calcium and phosphorus concentrations lead to mineralization defects developing rickets. Circulating levels of 25 (OH)-D are the best indicators of vitamin D levels. The expression of vitamin D receptor is present in small intestine, colon, osteoblasts, osteoclasts, activated T and B lymphocytes, pancreas, brain, heart, skin, gonads, breast, prostate and mono nuclear cells (15, 22).

Physiological aspects of vitamin D metabolism are presented in the figure 2 (13).

In addition to the role in bone mineral homeostasis, vitamin D has other non-calcemic actions such as thyroid function (17), insulin secretion (18), modulation of lymphocyte function and immune function (19), cellular differentiation and proliferation (23), effects on myocardial contractility (20), etc.

Calcitriol ($1,25\text{-}(OH)_2D_3$), major circulating form of vitamin D, exerts antineoplastic activity via genomic or non-genomic mechanisms in various cancers such as prostate, breast, colon, and blood cancers (29).

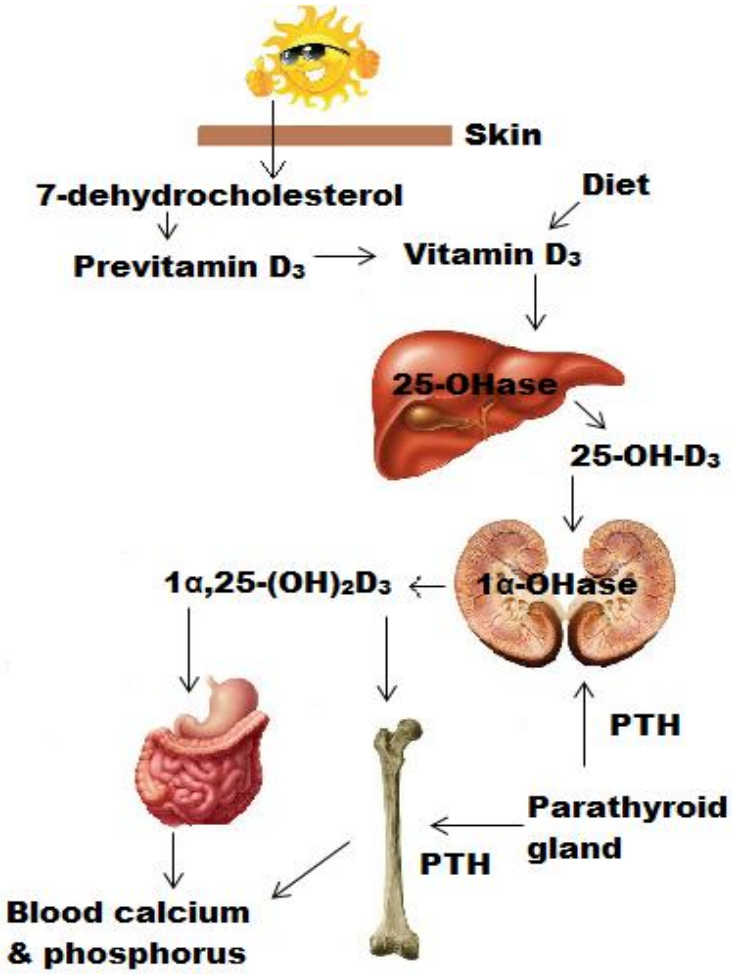


Figure 2: Schematic representation of Vitamin D metabolism

In genomic pathway gene transcription regulation is achieved by hetero-dimerization of vitamin D receptor (VDR) with nuclear retinoid X receptor (RXR). In non-genomic pathway calcitriol modulates the functions of calcium channels. Calcitriol mediates the transcriptional and post-transcriptional control of the gene expression in various cancers. Calcitriol induces cell differentiation in cancer cells, arrests proliferation and suppresses cell cycle and reduces cancer cell

survival time by regulating the transcription factors such as ubiquitous AP-1 complex. Non-genomic functions of vitamin D include intestinal calcium absorption, bone mineralization by acting of osteoblasts, opening of voltage gated calcium and chloride channels, activation of kinases and phospholipases (30, 31). Differentiation pathways of calcitriol vary in different cancer types. For example, in breast and prostate cancers, calcitriol exerts anti-proliferative and apoptotic effects (31-34).

The transcriptional and post-transcriptional control of the gene expression in various cancers may be mediated by vitamin D. Vitamin D receptor aids in transcription functions of vitamin D modulating the expression of genes involved in cell cycle. Calcitriol mediated differentiation effects may be cell specific. For example in monocytic cells, calcitriol induces differentiation in two phases. In the first phase expression of monocytic phenotype markers occur and this phase lasts for 24-48h while the cells continue in the normal cell cycle. Second phase involves in blocking G1 to S phase in cell cycle. In breast and prostate cancers, calcitriol exerts anti-proliferative and apoptotic effects on the cell lines (24-31). Administration of calcitriol in early stages exerted better anti-tumor activity than in later stages. Calcitriol functions via VDR regulating cell proliferation, apoptosis angiogenesis, and promotes differentiation in colorectal cancer (34). In ovarian cancer 1α,25-hydroxyvitamin D_3 induces apoptosis by destabilizing telomerase reverse transcriptase (21). Vitamin D increases vascular endothelial

growth factor (VEGF) in smooth muscles and upregulates mRNA of potent anti-angiogenic factor thrombospondin in human colon cancer cells (47). The mechanism underlying vitamin D mediated effects in cancer cell differentiation, proliferation, apoptosis and angiogenesis do not follow a particular pattern demonstrates heterogeneity in vitamin D effects or heterogeneity due to inherent cancer cell biology (33). Hence, it is suggests the potentiating action of vitamin D on anti-cancer effects of chemotherapeutic agents.

Vitamin D and Osteosarcoma: Characteristic knockdown of CYP27B1 in human OS cell lines resulted in proliferation (35); incubation with vitamin D resulted in promoted mineralization (36).

Vitamin D modulates gene transcription in osteoblasts producing matrix proteins such as osteopontin, osteocalcin, osteonectin play a key role in bone mass formation (33, 34). Hara K et al reported the oral administration of 1α-hydroxyvitamin D_3 inhibits tumor growth and metastasis in Dunn OS cell lines (35).

Vitamin D at concentrations from 10nM to 100nM induced differentiation in human OS cell lines 143B, SaOS-2 (33, 40). Expression of several differentiation markers such as osteocalcin, alkaline phosphatase, bone gla proteins increased in relation to dose of vitamin D (38). Osteocalcin, alkaline phosphatase (ALP) and bone gla proteins are directly related to degrees of osteogenesis indicating bone differentiation. Expression of differentiation markers is an important aspect for

calcitriol use in OS. Calcitriol treatment significantly reduced caspases activity in UMR 106 cell lines (37).

Cisplatin and Calcitriol in cancer: Cisplatin is the most commonly used drug to treat various types of cancers. Toxicity, tumor resistance and poor bioavailability are the undesirable features of cisplatin therapy. Cisplatin toxicities with high doses include neurotoxicity leads to hypokalemia, hypocalcemia and hypomagnesemia, ototoxicity and infertility in males (9). Resistance to cisplatin is the major concern in the treatment of cancers with cisplatin. Cisplatin acts by binding to DNA of the cancerous cell leading to lesions in DNA. Cisplatin resistance may arise due to copper transporters or because of intensified activation by thiol molecules such as glutathione or metallothionein. Cisplatin resistance in ovarian, cervical, lung and bladder cancer can be attributed to increased glutathione levels. Tumor suppressor protein p53 inactivation, survivin expression, decreased accumulation and apoptosis signaling proteins also lead to decreased cisplatin resistance (38, 39).

Calcitriol enhances the anti-neoplastic activity of various cytotoxic or chemotherapeutic agents. Ying yu et al studied calcitriol effects on various cancers in combination with chemotherapeutic agents such as cisplatin, carboplatin, doxorubicin, fluorouracil, gemcitabine, paclitaxel and docetaxel (31). Calcitriol potentiates cisplatin/carboplatin in breast and prostate cancer. Calcitriol pre-treatment sensitized squamous cell carcinoma cells to cisplatin therapy and

promotes cytotoxicity of fluorouracil in colon cancer. The mechanisms of action for enhancing the activity are different in each cancer (46). Combination of vitamin D with cytostatics reduced the inhibitory concentration (IC_{50}) values in cancers sensitive to vitamin D such as squamous cell carcinoma (44) breast (59), colon (47) and prostate cancers (42). Use of hypocalcemic vitamin D synthetic analogues in sensitization experiments is beneficial owing to their less toxic effects (35).

Moffatt et al reported elevated growth inhibition of prostate cancer cells was observed in the combination therapy of higher concentrations of vitamin D and lower concentrations of platinum drugs (42). Potentiating effect of calcitriol on cisplatin was investigated by Hershberger et al. They reported the role of mitogen activated protein kinase kinase (MEKK-1) pathway mediates cisplatin induced apoptosis by calcitriol pretreatment in squamous cell carcinoma (43). From the above literature we can infer, calcitriol and cisplatin act synergistically in certain cancers.

Resistance to chemotherapy, toxicities of the chemotherapeutic agents and high recurrence rates in OS put forth a challenge of sensitization of cancerous cells in order to provide improved treatment regimes. Commonly used treatment regimes are not effective in leukemia with myeloid/lymphoid or mixed lineage leukemia (MLL) gene. Hence, inducing differentiation is a useful strategy in such cases. All trans retinoic acid (ATRA) and calcitriol were used to induce differentiation in

combination with 5-aza-2'- deoxycytidine therapy. Both ATRA and calcitriol potentiated the effect of 5-aza-2'- deoxycytidine in MLL gene chemoresistant leukemia (45). Sensitization may induce apoptosis along with differentiation in various cancers. Calcitriol sensitization induces caspase independent apoptosis in colon and breast cancer cells. In breast cancer cells, the production of enzymatic protein cathepsin B and tumor necrosis factor receptor I (TNF-RI) are increased. The mechanisms of differentiation induced by vitamin D and TNF-α are different, combination of these agents enhanced apoptosis (46, 47). Sensitization of OS cells to chemotherapy is an ongoing process in OS management. Literature suggests the sensitization of OS cell lines with bone anabolic agents such as statins for example; atrovastatin and bisphosphonates for e.g. zoledronic acid are effective in enhancing the chemotherapeutic response (49, 51). Olivia et al found atrovastatin induced sensitization of OS cell lines in combination with doxorubicin treatment decreased cell viability and migration (49). Zou et al reported sensitization of OS cell line is by down regulating an apoptosis inhibitor protein, survivin (50).

The mechanism for the sensitization by calcitriol may be via regulation of caspases. Caspases are cysteine-aspartate proteases with an important role in apoptosis. Regulation of caspase dependent apoptosis with calcitriol sensitization was reported in squamous cell carcinoma, prostate and breast cancer (31, 37, 43, 44, 50).

Matrix metalloporteins: Matrix metalloproteins (MMP) are zinc dependent proteases, play a vital role in cell proliferation, migration, invasion, differentiation and apoptosis by degradation of extra cellular matrix (12, 29, 54). There are more than 20 types of MMPs and role of MMPs in tumors are well studied (61). Several MMPs are over expressed in bone and cartilage, for example MMP-9 and MMP-13. Over expression of MMP-12 in colon cancer, MMP-3 in breast and prostate cancer, MMP-2 in bone, prostate and squamous cell carcinoma are reported; altered rate in survival and metastasis was attributed to MMP-9 in prostate and OS (52-59). Down regulation of MMP-1 and MMP-9 by calcitriol and its analogues in breast cancer cell lines was reported (58, 59). Modulation of MMP-13 and MMP-1 expression by calcitriol in osteoblast like cells was reported (56, 60-62). Kimura et al reported role of MMP-1 in 143B cell invasion, and pulmonary metastasis of OS (63). However, modulation of expression of different MMPs by calcitriol is not clearly investigated and is a subject of intense research in understanding OS pathobiology and disease management.

Gaps in research:

Calcitriol is a potent differentiation inducing agent in OS. There not much information available in the current literature in support of calcitriol and OS treatment. The mechanism for resistance in OS needs to be addressed and thoroughly investigated for devising effective chemotherapy. There is emphasis on calcitriol sensitization in breast, colon and prostate cancers in literature. The anti-apoptotic activity of calcitriol should be studied further and the mechanism of sensitization should be made clear.

Differentiation and anti-proliferation effects of calcitriol are well established. Recent research shows sensitization of OS cell lines with bone anabolic agents such as statins, bisphosphonates enhances the anti-neoplastic activity of chemotherapeutic agents. The aim of the present study is to evaluate the effects of sensitization of OS cell lines with calcitriol prior to cisplatin treatment and examine its effects on migration, invasion and apoptosis.

METHODS

Setting: All experiments were carried out at The University of Kansas Medical Center (KUMC) in lab 1006 Briendenthal Annex and the Department of Dietetics and Nutrition.

Ethics: This study qualifies for the exemption from Human Subjects Approval as the cell lines used in these *in vitro* experiments are considered as secondary data. The cell lines used in this study contain no identifying marker, available publicly and are obtained from American Type Culture Collection (ATCC).

Materials:

Cell lines: Osteosarcoma (OS) cell lines 143B parental (143B-P), 143B metastatic murine (143B-MM) and MC3T3-E1 (mouse pre-osteoblast) were used in the study. 143B cell lines are K-ras and P53 gene mutated. 143B-MM is metastatic version of 143B, obtained from metastatic lung lesions of *in vivo* bioluminescent orthotopic OS mouse (BOOM) model. Both the OS cell lines are highly proliferative and metastatic.

Cell Cultures: Osteosarcoma and osteoblast cells were cultured and maintained in Dulbecco's' Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 5% Penicillin/Streptomycin, 5% Amphotericin, 5 % L-glutamine and 5% non-essential amino acids. The cells were maintained in a

humid incubator at 37^{o}C with 95% air and 5% CO_2. Medium was changed for every 3 days.

Reagents: Calcitriol was purchased from Enzo Life Sciences International Inc., (Plymouth Meeting, PA) and cisplatin 1mg/ml was purchased from KUMC pharmacy. Stock solutions of calcitriol was prepared in ethanol and cisplatin in dye free DMEM. Calcitriol aliquots were stored at -20^{o}C and cisplatin at room temperature.

Data collection procedures: For the experiments confluent OS cells and osteoblasts were harvested by Trypsin digestion (trypsinization) of the adherent cell cultures. Following trypsinization, the cells were counted using trypan blue in hemocytometer. Trypan blue is a dye used to stain the dead/non-viable cells. Viable (unstained) and non-viable (stained) cells were counted in three different quadrants of hemocytometer (n=3). A defined number of cells were seeded in 96-well/24well/6-well plates or T75 flasks. The average number of cells per ml was calculated using the following formula:

Cells per ml = Average number of cells (in three quadrants) x 2500 x 10

Where 2500 is the area and 10 is the dilution factor.

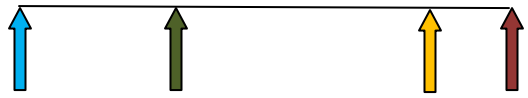
Cell viability assay: CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) and colony formation assay are used for the evaluation of cell

proliferation and viability. MTS assay is a colorimetric method to determine the number of viable cells. Mitochondrial activity of the cells when MTS tetrazolium compound is converted into formazan on reaction with dehydrogenase enzyme is measured. Viable cells convert the MTS to formazan, whereas non-viable/dead cells do not. The IC_{50} value of cisplatin for the above OS cell lines is determined from dose response curves using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. Ten thousand cells per well were plated in 96-well plates, after 24h when the cells were confluent calcitriol and cisplatin were added. Different concentrations of cisplatin are represented as C1 (50nM), C2 (100nM), C3 (250nM) and C4 (500nM) were used in each row. After 48h of incubation with calcitriol/cisplatin the medium was aspirated, 100µl of fresh medium and 20µl of MTS reagent were added. Cell viability was evaluated after 1hour of incubation using a microplate reader at 490nm.

Control	Calcitriol 100nM	C1 50nM	C2 100nM	C3 250nM	C4 500nM

MTS assay

C1, C2, C3 and C4 represent four different concentrations of cisplatin.



(A) (B) (C) (D)

(A) Represents cell plating

(B) Addition of cisplatin/calcitriol after 24h

(C) Addition of MTS reagent after 48h

(D) Terminating the reaction and reading absorbance after 1h.

The MTS assay was also used to evaluate sensitization effects of calcitriol apart the cisplatin dose response. Ten thousand cells per well were plated in 96-well plates and 100nM calcitriol was added after 24h. After 24h of addition of calcitriol, four different concentrations of cisplatin were added to the cells and incubated for 48h. The cells in 96well plate are treated with MTS reagents and read at 490nm in a spectrophotometer.

Cntrl	Calcitriol 100nM	Cal. + C1 Cal.@100nM C1@50nM	Cal. + C2 Cal.@100nM C2@100nM	Cal.+ C3 Cal.@100nM C3@250nM	Cal. + C4 Cal.@100nM C4@500nM

Cal. stands for Calcitriol and C1, C2, C3 and C4 for different concentrations of cisplatin.

Colony formation assay: It is a gold standard technique used to determine the cytotoxic effect of the chemotherapeutic agent. For this assay, 10,000 cells per well were seeded in 6-well plates and calcitriol (100nM) was added at confluence (48h). After 48h exposure to calcitriol, cisplatin was added and the cells were incubated for 48h. The medium was aspirated and cells were washed three times with phosphate buffer solution (PBS). The cells were fixed with

paraformaldehyde and stained with crystal violet. The stain was extracted by 1% acetic acid and quantified at 570nM.

Control	Calcitriol 100nM	Calcitriol + C3 Cal.@100nM C3 @250nM	Calcitriol + C4 Cal. @ 100nM C4 @ 500nM
Control	Calcitrol 100nM	C3 250nM	C4 500nM

C3 and C4 for different concentrations of cisplatin

Migration assay: The effect of calcitriol on migration of OS cell lines was studied using Boyden-Chamber method. Osteosarcoma and osteoblast cells were pre-treated with calcitriol for 72h. Calcitriol pre-treated and untreated (control) cells were plated in the inserts placed in 24-well plates. Five thousand cells were plated in the inserts and after 24h; the cells were fixed with ethanol and stained with crystal violet. The number of crystal violet stained cells were quantified colorimetrically at 560nm following extraction with 1% acetic acid.

Control	Calcitriol treated 100nM
Control (in the insert)	Calcitriol treated 100nM (in the insert)

Invasion assay: Collagen and matrigel were used to study the invasive effects of OS and osteoblast cells. Matrigel was used to simulate the tumor micro-environment and collagen for bone micro-environment. The inserts were coated

with collagen/matrigel and incubated for 30mins. After collagen/matrigel polymerization, coated inserts were conditioned with serum free medium for 1h. Five thousand cells per insert were plated and after 48h invaded cells on the bottom of the insert were stained with crystal violet subsequent extraction and detection at 560nm was carried out.

Matrix metalloprotein spectrophotometric assay: Matrix metalloproteins (MMPs) are membrane associated zinc endopeptidases with a significant role in wound healing, arthritis and cancer. SensoLyte Generic MMP assay kit was used for MMP activity evaluation. Cells treated with and without calcitriol were washed with HBSS; 10µg/ml of Trypsin was added and incubated for 1h. Trypsin inhibitor was added after 1h at 100µg/ml concentration for 15mins. MMP substrate solution, MMP inhibitor and diluents were prepared. The test components were added into the microplate along with the controls in triplicates. The plate was read for kinetics at 37^{o}C absorbance at 412nm and data is recorded every 10mins for 1h. Glutathione reference standard was used for instrument calibration with concentrations 100, 50 and 25 µM.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Total RNA isolation: For RNA isolation, 2.5×10^4 cells were plated in 6-well plate and calcitriol 100nM was added to the three wells at confluence having control and calcitriol treated samples. After 72h of calcitriol addition total RNA

was isolated from the cells by Qiagen RNeasy mini kit and stored at -20^{o}C. The ratio of absorbance at 260/280 was used to measure the purity of RNA and the samples with ratio of approximately 2 and nothing less than 1.85 were used for PCR.

Reaction mix for PCR:

Component	Quantity
Sybr green	12.5µl
Primer A	1.25µl
Primer B	1.25µl
Reverse Transcriptase	0.25µl
Template RNA	Variable (100-500ng)
RNA free water	Up to 25µl

The real time PCR was performed using iCycler (Bio-rad) at Dr. Rowe's lab. (Dr. Peter Rowe, Kidney Institute, University of Kansas Medical Center). The program used for PCR was:

Step	Time	Temperature
Reverse transcription	30mins	50^{o}C
Initial PCR activation	15mins	95^{o}C
Denaturation	1min	94^{o}C
Annealing	1min	60^{o}C
Extension	1min	72^{o}C

Denaturation, annealing and extension steps were repeated for 40cycles with final extension time of 10mins at 72^{o}C.

Relative expression between samples was calculated using Livak's equation $X_n = 2^{-\Delta\Delta C}{}_T$. X_n is the relative amount of target genes and ΔC_T is difference between cycle threshold of the test gene (MMP-1) and reference gene (GAPDH).

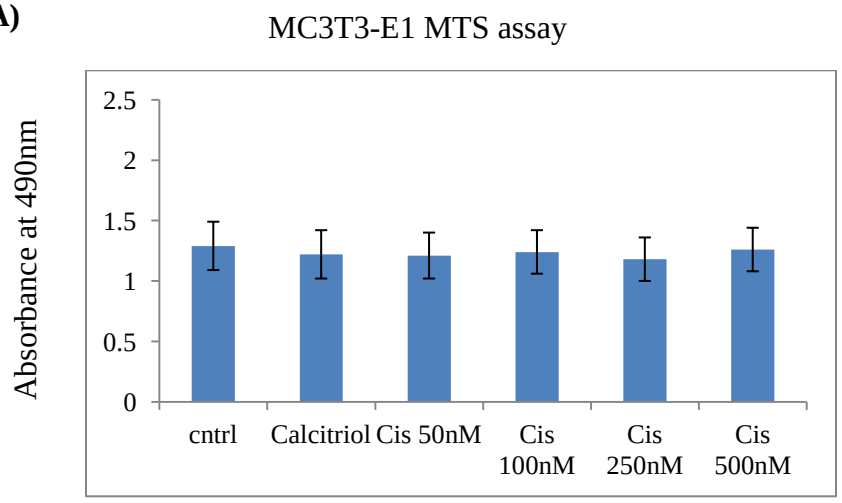
Statistical analysis: Microsoft excel was used for statistical analysis. Mean, standard deviation, variance and standard error was calculated for all the data. Paired t-test and single factor analysis of variance (ANOVA) were used to analyze the difference between control and experimental data. The P value of less than 0.05 was considered statistically significant.

RESULTS

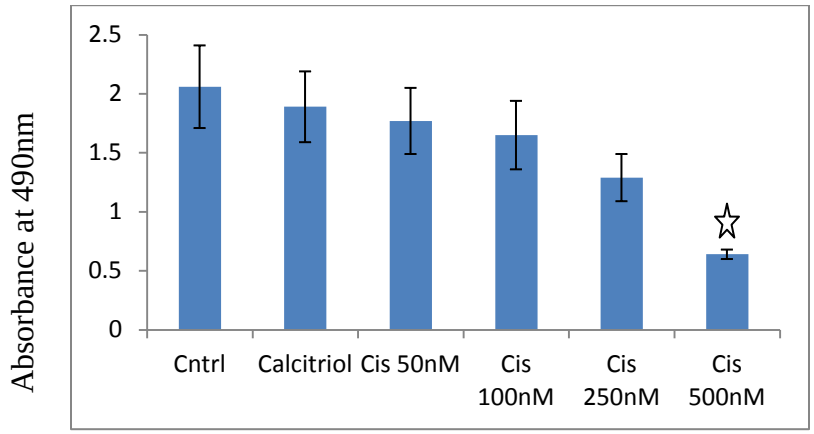
The purpose of this study was to investigate the sensitization effects of calcitriol on OS cells for cisplatin therapy. The effect of calcitriol on proliferation and migration of OS cells lines 143B-P, 143B-MM and MC3T3-E1, murine osteoblast control were analyzed. The cell line 143B-P is parental human OS cell line with K-ras and p53 mutations.

Cell proliferation was measured using MTS assay. The cells were cultured for 4 days with different concentrations of cisplatin to establish $IC_{50,}$ the doses of cisplatin required to inhibit half the cell population. The concentration of cisplatin required to significantly inhibiting the proliferation of both 143B-P and 143B-MM cell lines were 500nM. On the other hand, this dose of cisplatin was ineffective on control osteoblast cell line MC3T3-E1 (**Figure 3**).

(A)



(B) 143B-P MTS assay



(C) 143B-MM MTS assay

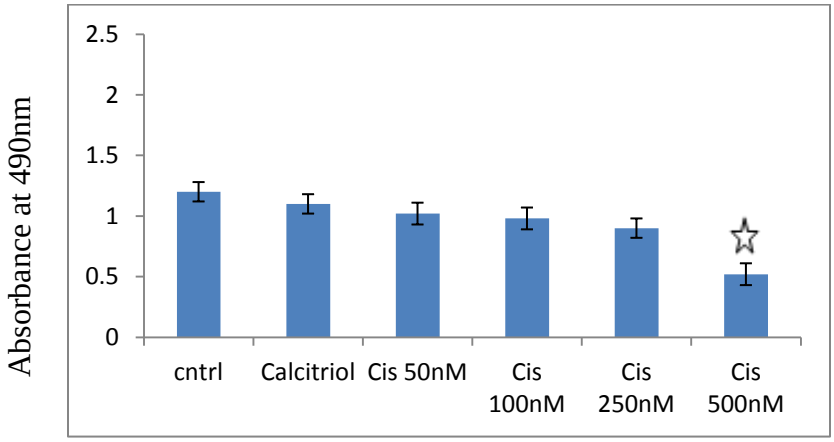
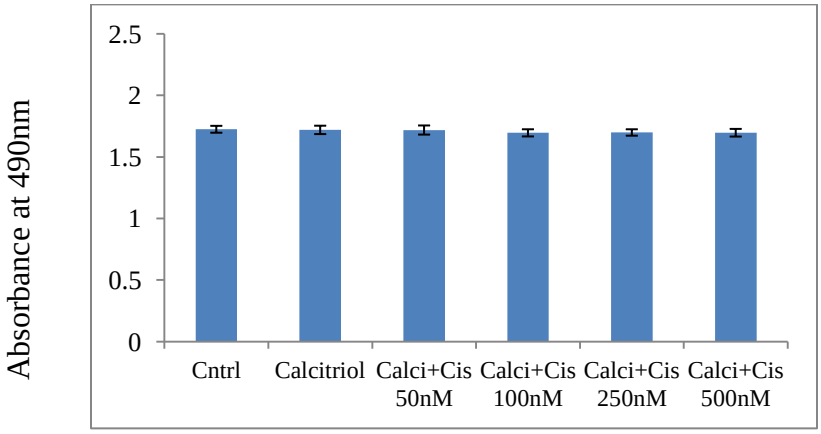


Figure 3: Dose response of cisplatin on the cell lines, (**A)** MC3T3-E1 **(B)** 143B-P and **(C)** 143B-MM. Cell proliferation measured after 96h treatment with 100nM calcitriol / varying concentrations of cisplatin. ☆Represents statistical significance with $P < 0.05$.

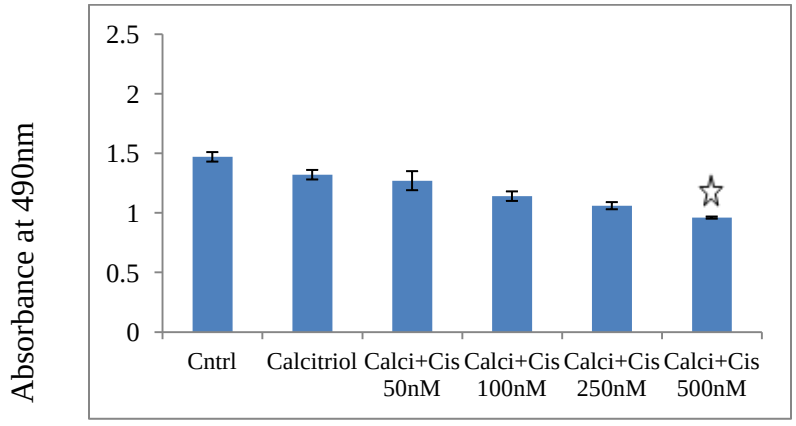
Sensitization effect of calcitriol on the OS cell lines for cisplatin therapy was studied using MTS assay and colony formation assay. Pretreatment of

OS cells with calcitriol did not have any significant sensitization effects for cisplatin therapy at all tested doses. There was no significant sensitization effect of calcitriol on either of the cell lines. (**Figure 4**)

(A) MC3T3-E1 MTS assay



(**B**) 143B-P MTS assay



(C) 143B-MM MTS assay

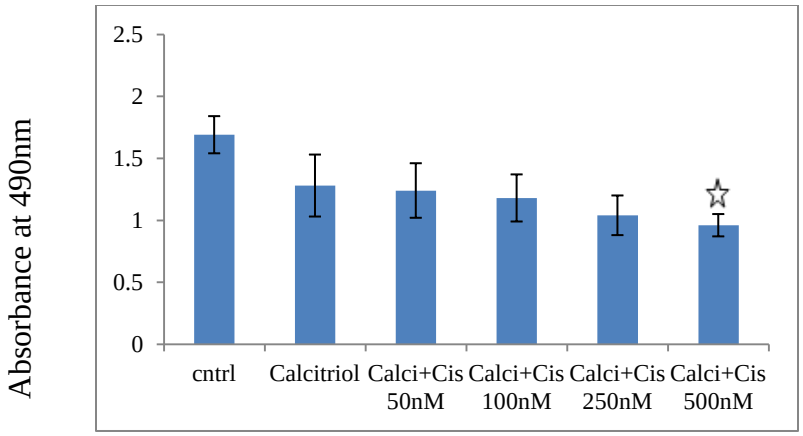
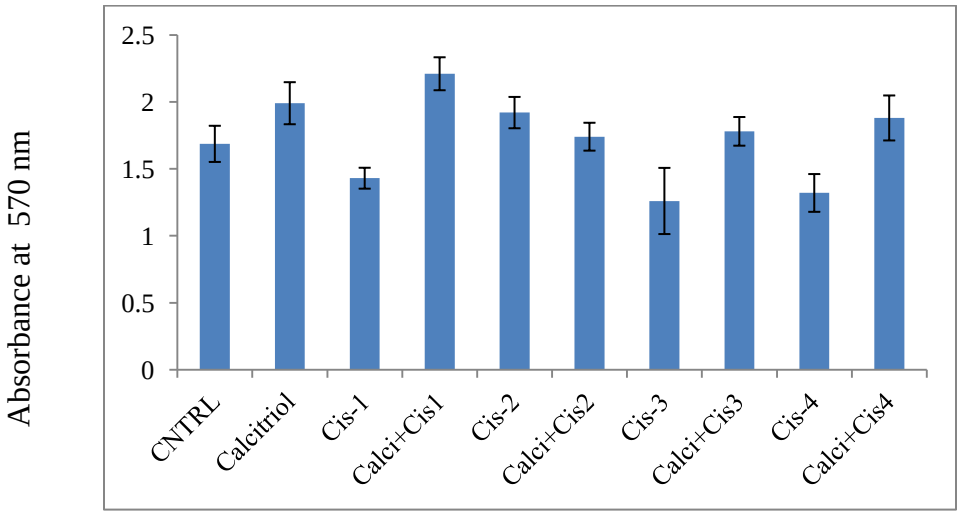


Figure 4: Sensitization effect of calcitriol for cisplatin therapy on the cell lines, (**A)** MC3T3-E1 **(B)** 143B-P and **(C)** 143B-MM. Cells were sensitized with 100nM calcitriol for 48h prior to cisplatin treatment and cell proliferation was measured after 48h treatment with varying concentrations of cisplatin. ☆Represents statistical significance with $P<0.05$. Significance was found in regard with cisplatin treatment at the highest dose but not for calcitriol's sensitization effects.

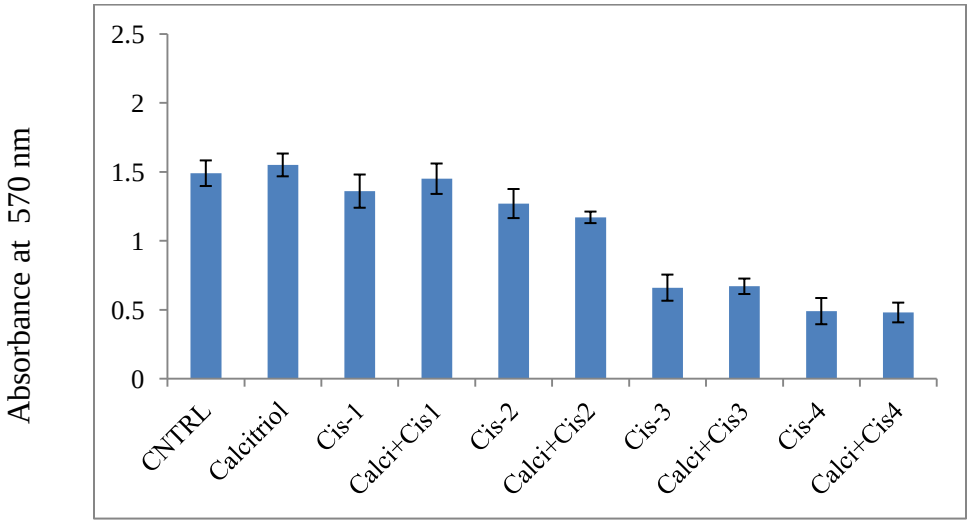
Sensitization effects of calcitriol were also evaluated using high doses of calcitriol such as 500nM and 1µM. Cisplatin dose of 500nM was used as it corresponds to IC_{50} in our studies. No significant sensitization effects were observed with either of the doses.

Colony formation assay, a gold standard technique for cytotoxicity determination. No significant sensitization effects of calcitriol were observed in either of the cell lines (**Figure 5).**

(A) MC3T3-E1



(B) 143B-P



(C) 143B-MM

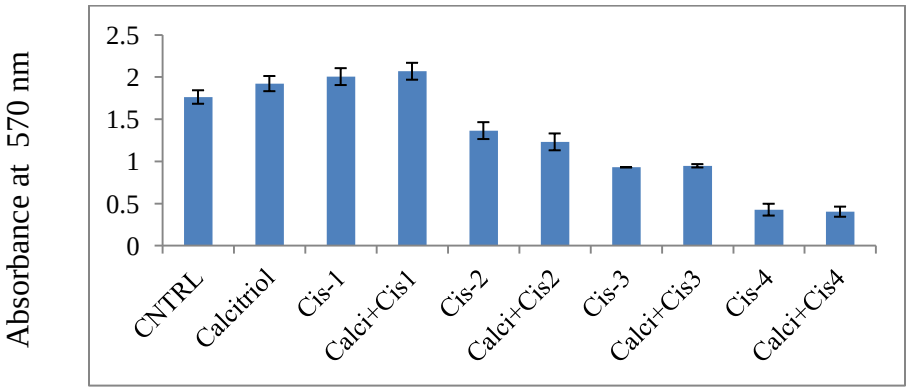
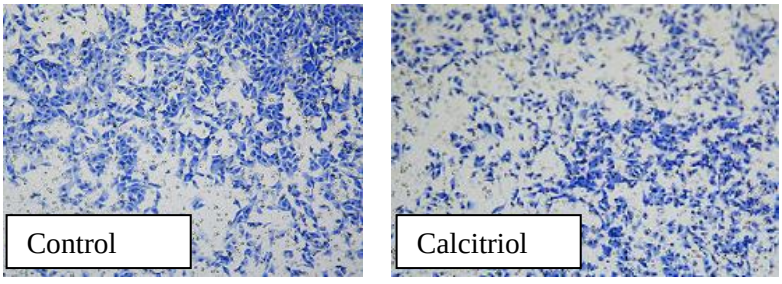


Figure 5: Effect of calcitriol on sensitization of (A) MC3T3-E1 (B) 143B-P (C) 143B-MM to cisplatin treatment by colony formation assay. Calcitriol at 100nM concentration was used and Cis-1= 50nM, Cis-2=100nM, Cis-3=250nM and Cis-4=500nM.

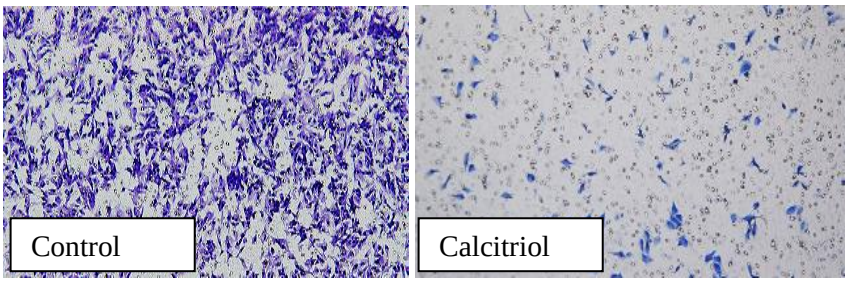
Migration and Invasion: Effects of calcitriol on migration and invasion of OS cell lines 143B-P and 143B-MM, and control osteoblast were investigated using Boyden-chamber method.

Calcitriol exerted a significant effect on the migration of 143B-P cell line in comparison with control ($P<0.5$). In 143B-MM and control osteoblast MC3T3-E1 cell lines, there was no significant difference between treated and untreated groups (**Figure 6**).

(A) MC3T3-E1



(B) 143B-P



(C) 143B-MM

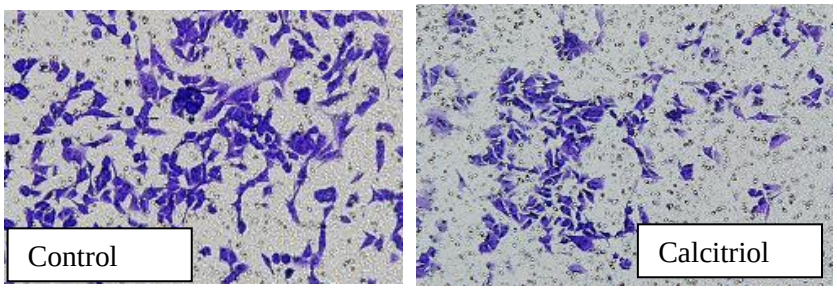


Figure 6: Representative photomicrographic illustration showing the effect of calcitriol on the migration of (A) MC3T3-E1 osteoblast cells (B) 143B-P and (C) 143B-MM. Control and calcitriol pretreated cells were plated in the inserts, incubated for 24hrs and stained with crystal violet for 20mins. Stained cell were imaged at 10X magnification and the bound stain was extracted with 1%acetic acid.

The migration data was quantified by extraction with acetic acid and absorbance was read at 570nm (**Figure 7**).

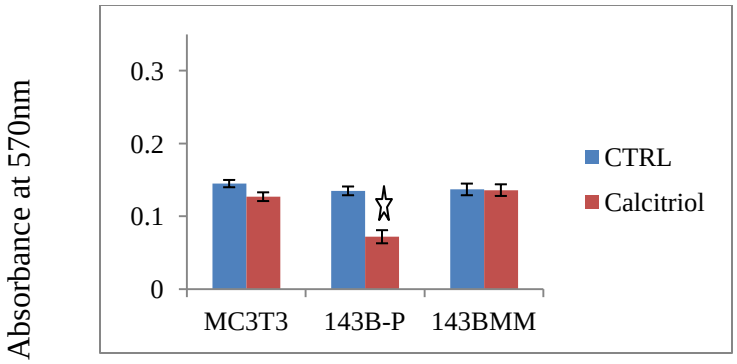
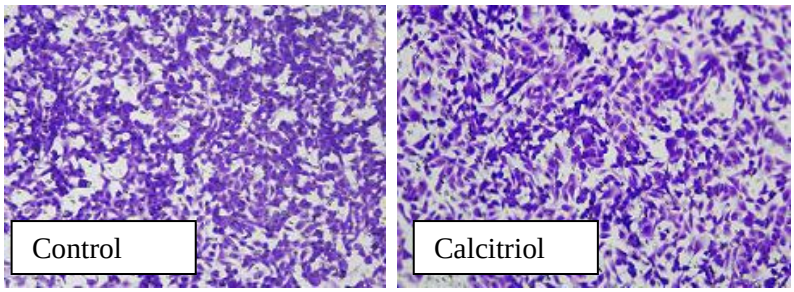


Figure 7: Effect of calcitriol on migration of osteoblast and OS cell lines. Control and calcitriol sensitized cells were cultured in the inserts for 96h; the inserts were stained with crystal violet for 20mins and the bound stain was extracted from the cells with 1% acetic acid. Absorbance of the extract was read at 570nm. ☆ Indicates statistical significance $P < 0.05$.

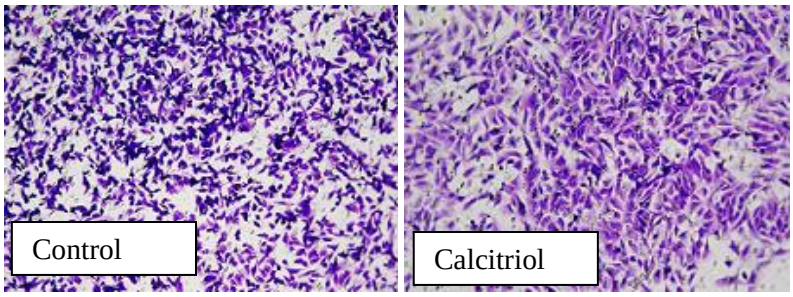
Invasiveness of the cell lines was studied using rat tail collagen type I and matrigel. Calcitriol treatment significantly inhibited invasion of 143B-P cells in matrigel. In collagen we observed decreased mobility of calcitriol treated control (untreated) osteoblast MC3T3-E1 (**Figure 8**).

(A) MC3T3-E1

(i) Matrigel

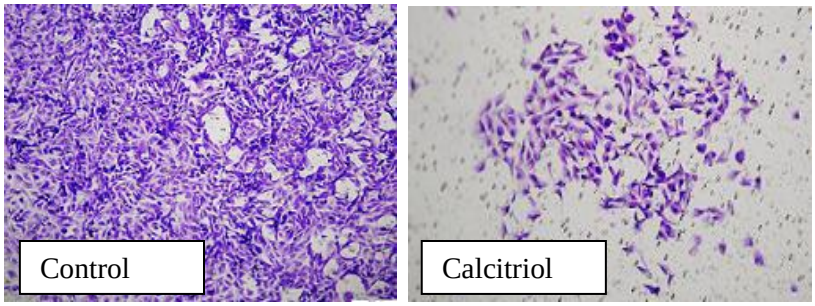


(ii) Collagen

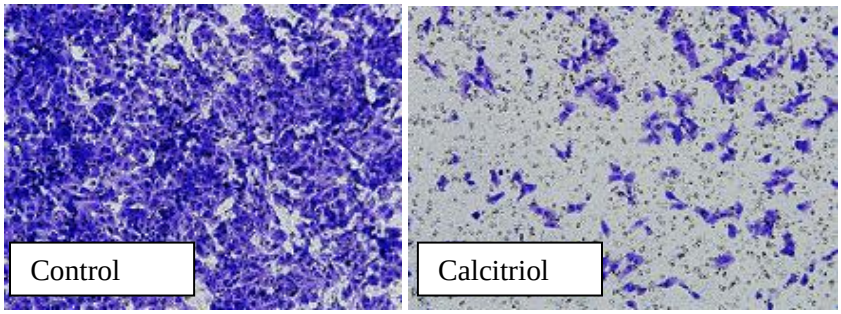


(B) 143B-P

(i) Matrigel

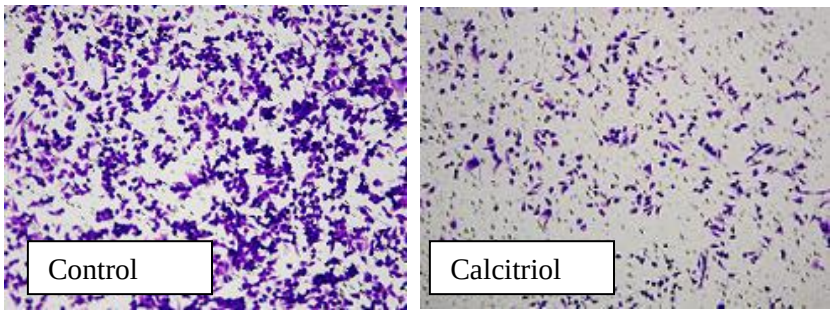


(ii) Collagen



(C) 143B-MM

(i) Matrigel



(ii) Collagen

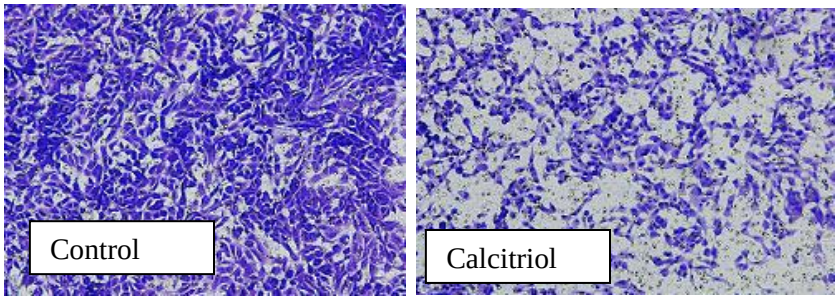
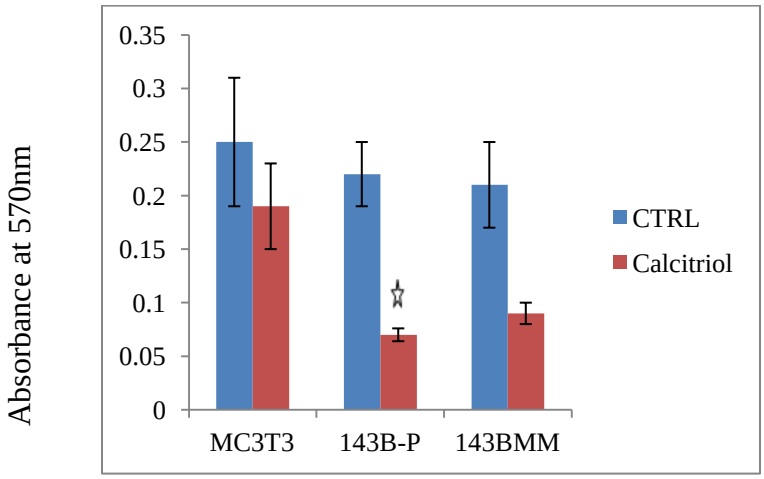


Figure 8: Representative photomicrographic illustration showing the effect of calcitriol on invasion of (A) MC3T3-E1 osteoblast cells (B) 143B-P and (C) 143B-MM though (i) matrigel and (ii)collagen. Inserts were coated with either matrigel or collagen for 1h and cells were plated and after 96h incubation inserts were washed and stained with crystal violet. The stained inserts were imaged at 10X magnification and stain was extracted with 1% acetic acid.

The crystal violet stains were quantified by extraction with 1% acetic acid and absorbance was read at 570nm. **(Figure 9)**

(A) Matrigel:



(B) Collagen:

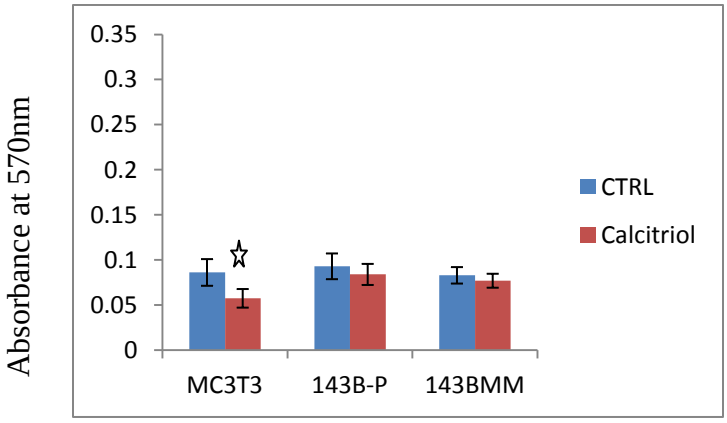


Figure 9: Effect of calcitriol on invasion of OS cells and osteoblasts in two different types of extracellular membranes molecules (A) Matrigel simulates tumor microenvironment and (B) Collagen, simulates bone microenvironment. Calcitriol sensitization decreased invasion of 143B-P cells in matrigel. ☆ Represents statistical significance with $P < 0.05$.

When collagen and matrigel membranes were compared for invasiveness of control and calcitriol treated OS and osteoblast cells, a significant

difference was observed between control OS cells whereas, in calcitriol sensitized OS cells we find no difference in invasion of cells in both the membrane simulators. In calcitriol treated osteoblast we observed a significant decrease in invasion in collagen membrane (**Figure 10)**.

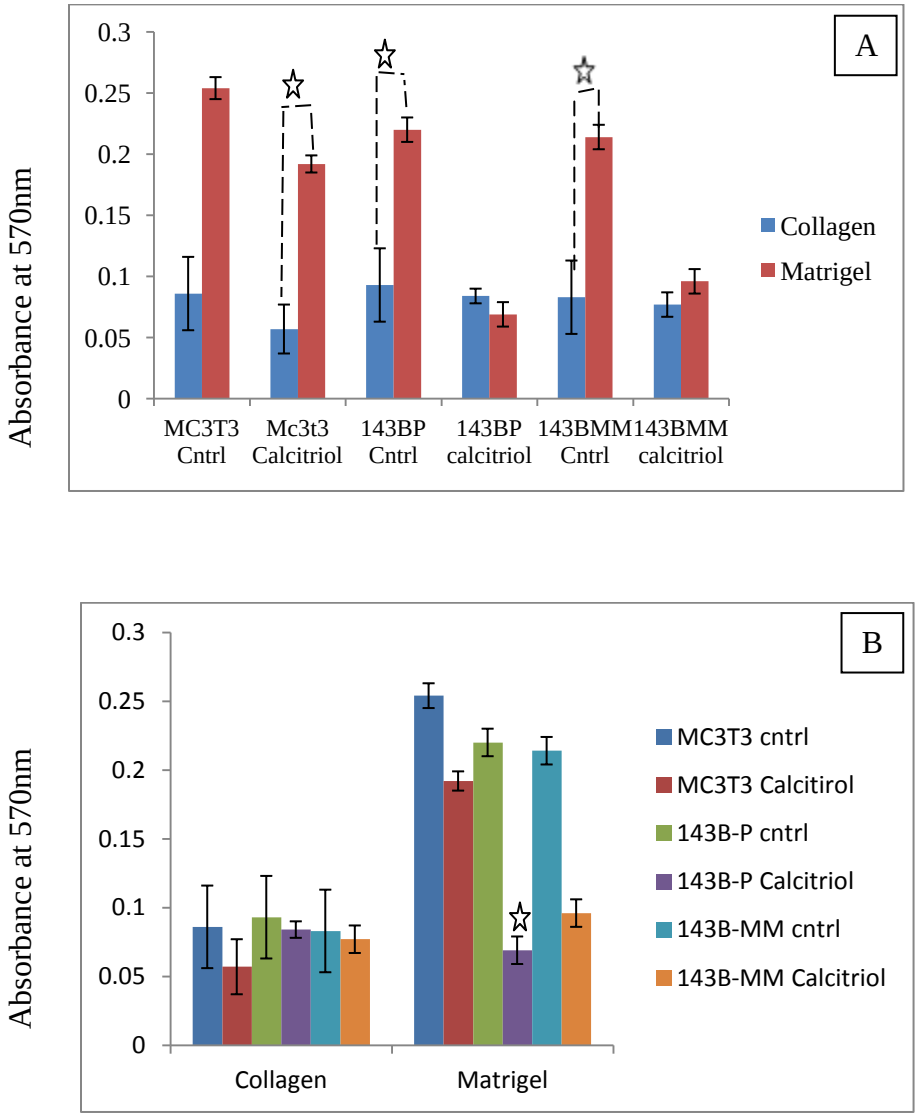


Figure 10: Comparison between matrigel and collagen membrane for invasiveness of (A) Control and calcitriol treated OS and osteoblast (B)

comparison between the membranes. ☆ represents statistical significance $P < 0.05$.

Matrix metalloprotein expression: Sensolyte generic MMP assay kit was used for spectrophotometric analysis of MMP 1,2,3,7,8,9,12,13 and 14. No significant differences were observed between calcitriol treated and untreated samples, though a slight decrease in calcitriol treated 143B-P was observed (**Figure 11)**.

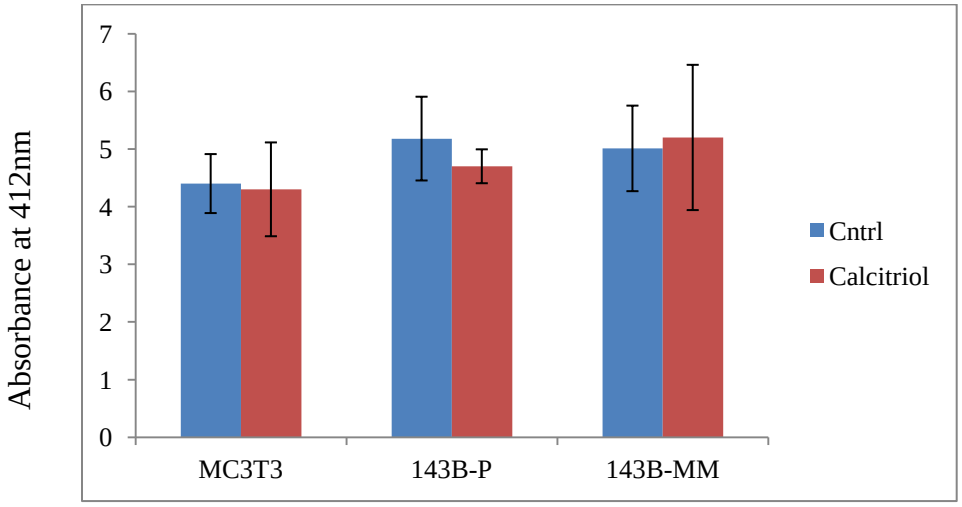
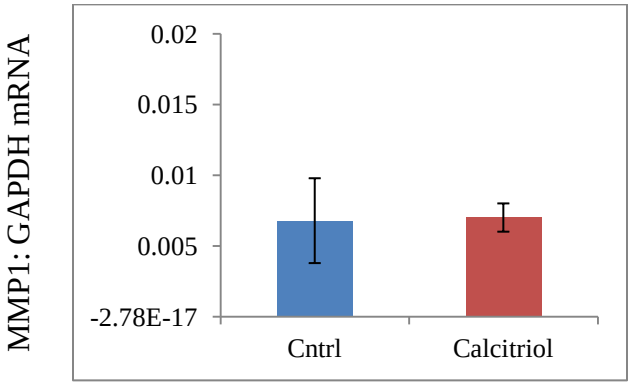


Figure 11: MMP activity kinetics analyzed with Anaspec generic MMP kit. Calcitriol pretreatment does not significantly decrease the MMP activity.

Real time RT-PCR assays were performed used two different sequences of MMP-1. Primer sequence-1 (PS1) was reported sequence by Kimura et al and primer sequence-2 (PS-2) is the sequence designed in our lab using Roche software (Appendix D). There were no significant differences in the expression of MMP-1 mRNA in calcitriol treated and control

(untreated) 143B-P cells with the reported sequence whereas, using our primer we did see a significant decrease in the expression of MMP-1 in calcitriol pretreated 143B-P cell line (**Figure 12)**.

(a) Primer sequence 1(PS1)



(b) Primer sequence 2 (PS2)

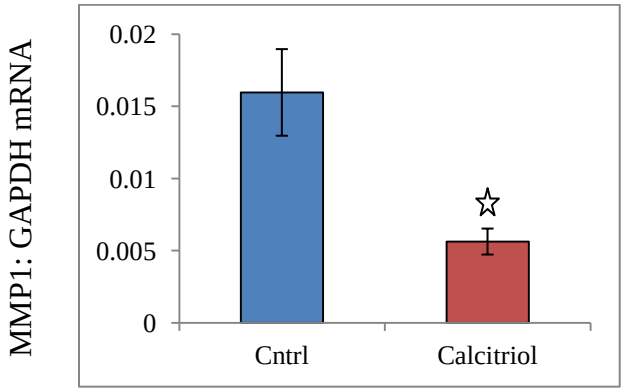


Figure 12: (**a**) Comparison of MMP-1 mRNA expression in control vs. calcitriol pretreated 143B-P OS cells using RT-PCR with reported PS-1 (**b**). Comparison of MMP-1 mRNA expression in control vs. calcitriol pretreated 143B-P OS cells using RT-PCR with PS-2 our design (N = 5 with triplicates every time). ☆ Indicates significance with $P < 0.05$.

DISCUSSION

Resistance to chemotherapy is the major concern in OS and sensitizing these cells should be beneficial strategy in the treatment protocol. Literature suggests calcitriol sensitization is an effective method in treating cancers such as breast, prostate and colon (15, 42, 47, 59). The previous findings in Dr. Garimella's lab suggest the presence of VDR and vitamin D responsive target genes in OS cells. In this study, we demonstrated calcitriol pretreatment for 48h does not sensitize OS cells 143B-P or 143B-MM for cisplatin therapy using MTS and colony formation assays. Inhibitory concentration (IC_{50}) of cisplatin was established by dose response MTS assay with all the three cell lines (Fig. 2A, 2B, 2C). Cisplatin's IC_{50} for 143B-P and 143B-MM was 500nM and at this dose osteoblast cells remained unaffected. Cisplatin concentrations ranging from 100ng to 20μM were used in different variety of OS cell lines such as U2OS and SaOS-2 (49, 51). High dose requirements of cisplatin may be due to the different mutational backgrounds or inherent heterogeneity among subpopulations of OS cells. SaOS-2 and U2OS are p53 null, whereas, 143B is K-ras gene mutated. Calcitriol sensitization occurs via arresting G_1/ G_2 phase in cell cycle in prostate cancer (42, 58); increasing caspase activation in colon cancer (47); and increasing mitogen activated protein kinase kinase expression in squamous cell carcinoma (43). In our studies no sensitization effect of calcitriol was observed in 143B OS cells. Higher doses calcitriol, 500nM and 1000nM (1μM) were also studied and

no sensitization effect was observed in OS cells. Calcitriol pretreatment for 48h was not appropriate time period to observe sensitization effects on OS cells. Other pretreatment times such as 72h and 96h need to be studied to obtain comprehensive data for the effects of sensitization.

Using the gold standard method, i.e. colony formation assay we examined the sensitization effect of calcitriol on OS cells. In accord with MTS assay results we observed no sensitization effect of calcitriol pre treatment in OS cells. The increase in proliferation as seen in Cal+C1, Cal+C3 and Cal+C4 vs. C1 or C3 or C4 (cisplatin alone) treatments for MC3T3-E1 cells is most likely due to rapid proliferative response of osteoblasts to calcitriol pretreatment, during the initial growth phase (30,31).

The cell line 143B-MM was obtained from the lungs of mice by inducing OS with 143B-P cell injection after metastasis. Though the origin of both 143B-P and 143B-MM cell lines is human OS, 143B-MM and 143B-P might differ biologically. These biological differences in the cell lines may account for the variation in migration of OS cells. 143B-MM cell characterization would be beneficial in understanding the mechanism of migration and invasion and response to chemotherapeutic/tumor modulating agents.

Calcitriol mediated different effects on migration of 143B-P and 143B-MM OS cells. While calcitriol exerted a significant effect on 143B-P cells, no significant effect was observed in 143B-MM and MC3T3-E1 cell lines.

The effect of calcitriol on the invasiveness of the OS cells lines was studied in matrigel and collagen. Matrigel is a commercially available reagent and it mimics the extracellular matrix of the tumor micro environment, thus providing the cancer cells with chemoattractants and growth factors such as epidermal, insulin like and fibroblast growth factor (66) for optimum growth. Collagen is the most abundant protein in mammals and a major component of connective tissue helps in protecting the cell integrity. Type 1 collagen simulates bone micro environment and acts as a chemotactic agent (67, 68). In matrigel, untreated OS and osteoblast cells along with calcitriol treated osteoblast cell invasion was significantly increased when compared to collagen. This may be attributed to the presence of growth factors in matrigel.

Calcitriol pretreatment significantly decreased invasion of 143B-P OS cells in both collagen and matrigel. In 143B-MM cell line the decrease in invasiveness upon calcitriol pretreatment was not significant. Increase in sample size for 143B-MM may yield significant results.

We hypothesized calcitriol sensitization regulates MMPs as MMPs are key mediator of cancer cell migration and invasion (58, 59, 61). Previous studies have demonstrated the importance of MMP-9, MMP-13 and more recently role of MMP-1 is evident in stimulating OS migration, invasion and metastasis (53, 60, 63) there by suggesting MMPs as important biomarkers and potential therapeutic targets in OS disease management. Kimura et al (63) reported enhanced

expression of MMP-1 in 143B, and the role of c-jun and Fra-1 in inducing MMP-1 expression.

Calcitriol pretreatment of OS cells did not regulate MMP activity (Fig 11).

The failure to detect MMP activity in calcitriol treated and untreated 143B-P cells using the MMP generic kit may be due to the presence of an unknown inhibitor in the reaction mixture containing crude OS cell extracts prepared from trypsin digestion. Hence, we used real time PCR to detect the expression of MMP-1 mRNA. MMP-1 was reported to be abundantly expressed in 143B OS cell lines (63). Previously reported Kimura et al sequence and a sequence designed by our lab were used to evaluate the expression of MMP-1 in calcitriol treated and untreated 143B-P OS cell line. In RTPCR experiment we used only one OS cell line 143B-P as the significant differences between control and calcitriol treatments in migration and invasion studies were evident in only143B-P cell line. In RT-PCR experiment calcitriol treatment did not affect the expression of reported sequence of MMP-1 whereas, with the PS-2 we see a significant decrease in MMP-1 expression on calcitriol treatment in 143B-P OS cell line (Fig 12). This suggests the role of other proteins such as MMP-9, MMP-13 and MMP-28 or CXCR4 in vitamin D mediated effects on the migration and invasion of 143B-P need to be investigated.

Conclusion:

This study investigated the effect of calcitriol pretreatment on 143B-P & 143B-MM OS cells and MC3T3-E1 osteoblast control. Though there are reports suggesting calcitriol sensitization for chemotherapy in different cancers, we did not find any sensitization effects of calcitriol on OS cells.

Calcitriol pretreatment was effective in inhibition of migration and invasion of 143B-P cells but not in 143B-MM cells. The effect of calcitriol on MMP1 mRNA expression was variable. With the reported primer sequence no significant difference in calcitriol induced changes in MMP1 expression was observed where as with the newly designed primer sequence, we observed a statistically significant change in the MMP-1 expression of calcitriol treated 143B P OS cells.

Limitations:

This an *in vitro* study where tumor micro environment complexity is compromised. Artificial substrates for physiological fluids such as FBS or reagents such as matrigel for simulating cytokines and growth factors present in tumor microenvironment are used. Heterogeneity in cancer cells can induce variable responses in terms of sensitization and chemotherapy. For sensitization experiments we used a single time point (48h) of calcitriol pretreatment. Increase in the exposure time to calcitriol might sensitize the OS cells for chemotherapy. Expression of MMP is regulated by various factors such as growth factors, cell-cell and cell-extra cellular membrane interactions. In migration and invasive

studies collagen and matrigel might have contributed for the regulation in MMP expression along with calcitriol. The sample size was small; replication of the experiments for MMP activity assay with protein measurement should be carried out. We analyzed only MMP-1 using PCR, evaluation of other MMPs should be done in order to understand the mechanism for migration and invasion.

Future directions:

In vivo studies are necessary to understand the role of calcitriol in inhibiting the tumor progression and metastasis in OS. Additional time points such as 72h and 96h should be included for complete evaluation of sensitization effect of calcitriol for chemotherapy in OS cell lines. Inclusion of more OS cell lines especially those differing in their mutational background will provide important information regarding calcitriol sensitization on chemotherapeutic response. The role of other proteins such as MMP-9, MMP-13 and MMP-28 or CXCR4 in vitamin D mediated effects on the migration and invasion of 143B-P need to be investigated.

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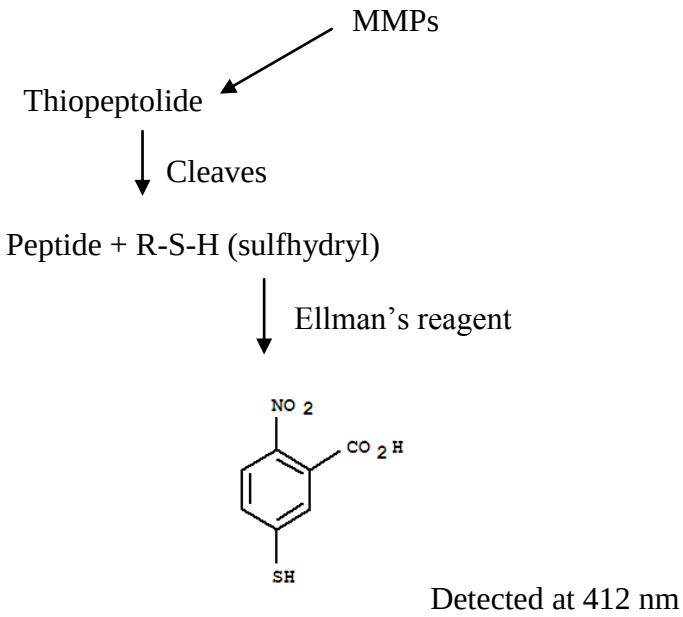
APPENDIX

APPENDIX A

Principle of MMP spectrophotometric assay:

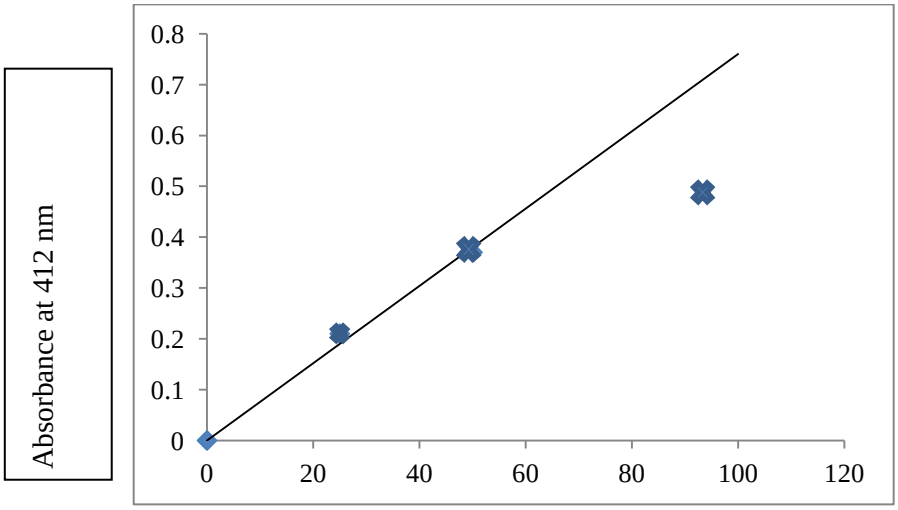
- Can be used to screen MMP inhibitors and inducers
- Chromogenic substrate, a thiopeptolide is cleaved by MMPs releasing sulfhydryl group.
- The sulfhydryl group reacts with Ellman's reagent (5,5'-dithiobis92-nitrobenzoic acid)
- Final product of this reaction is 2-nitro-5-thiobenzoic acid, can be detected at 412 nm.



APPENDIX B

Glutathione reference standard plot

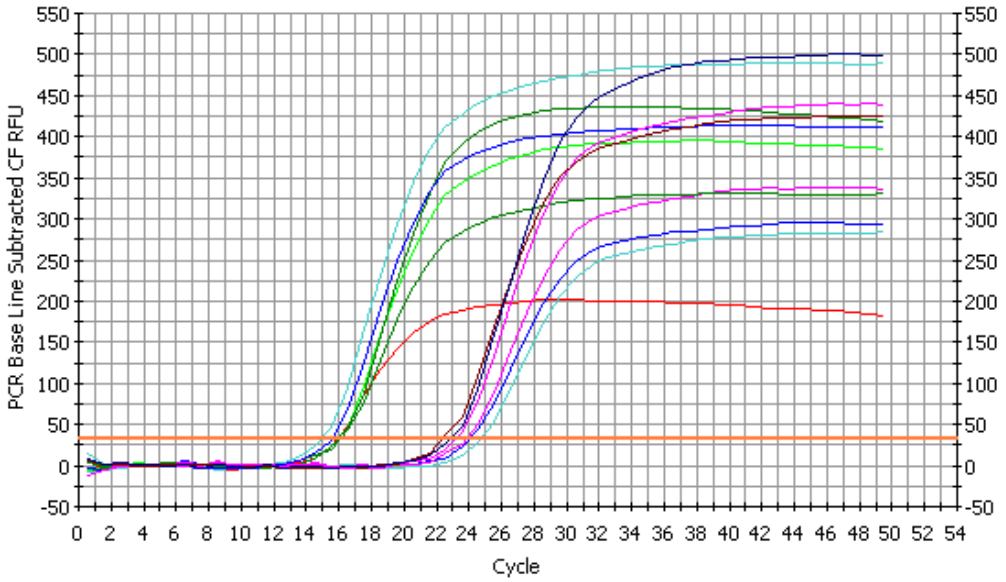
Used for instrument calibration provided in MMP detection kit.



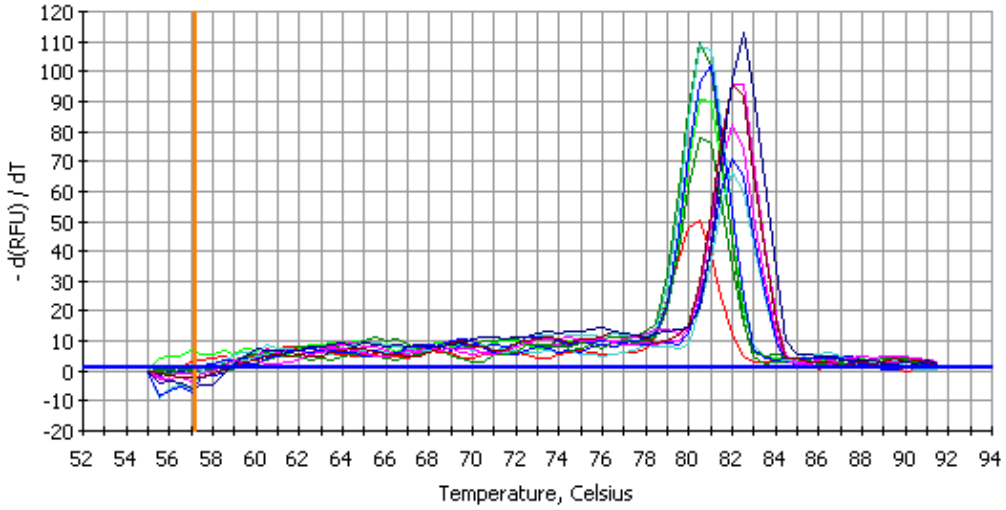
APPENDIX C

Reverse transcriptase polymerase chain reaction representative cycle graph and melt curve

PCR Amp/Cycle Graph for FAM-490



Melt Curve Graph for FAM-490



APPENDIX D

MMP-1 primer sequences

Reported (PS1)

Forward: 5’-GGTGCCCAGTGGTTGAAAAAT-3’

Reverse: 5’-CATCACTTCTCCCCGAATCGT-3’

Designed (PS2)

Forward: 5’-GCACAATCACAGCTCAATGC-3’

Reverse: 5’-TCATGCACCTGTAGTCCTAGTCC-3’

GAPDH primer sequence (STANDARD)

Forward: 5’-GAGTCAACGGATTTGGTCGT-3’

Reverse: 5’-GACAAGCTTCCCGTTCTCAG-3’